Effect of Antifungal Agents on Biological Fitness of *Lygus hesperus* (Heteroptera: Miridae)

JANET ALVERSON[1] AND ALLEN C. COHEN

Biological Control and Mass Rearing Research Unit, USDA, Agricultural Research Service, Mississippi State, MS 39762

J. Econ. Entomol. 95(2): 256–260 (2002)

ABSTRACT Artificial diets have become important components of rearing systems for insects that are used for research purposes and in commercial production. Because the rearing conditions for insects also provide ideal settings for mold growth, antifungal additives are often used to reduce diet contamination. However, the antifungal agents must not only be effective in mold suppression, they must also be safe to the target insects of the rearing programs. The toxicity of five commonly used antifungal agents (benzoic acid, formalin, methyl paraben, propionic acid, and sorbic acid) was tested using diet bioassays on *Lygus hesperus* Knight, and the effect on biological fitness was measured. Biological fitness was defined as total number of survivors, mean biomass (dry weight) accumulated per cage over the total treatment period, egg production, time to adult emergence, and time to start of egg laying. Methyl paraben and formalin were found to have significant negative effects on these measurements of biological fitness. Challenge tests to determine the ability of the antifungal agents to suppress mold growth when inoculated into the diet medium are currently in progress.

KEY WORDS *Lygus hesperus*, plant bugs, mass rearing, antifungal, artificial diet

THE WESTERN TARNISHED plant bug, *Lygus hesperus* Knight, is a very destructive and economically important pest in North America, particularly in the southwest (Clancy 1968). It feeds on a wide variety of seed and fruit crops such as alfalfa, beet, carrot, cotton, peach, strawberry, and bean (Strong 1970, Tingey and Pillemer 1977). The need for new management strategies for this pest has become apparent due to its extremely broad host range, and to its insensitivity to many conventional chemical control agents (Hedlund and Graham 1987). Laboratory research on management strategies such as biological control, sterile insect release, plant breeding, or genetic engineering of target crops, usually demands high numbers of high quality insects. Debolt (Debolt 1982) reported the first artificial diet to allow continuous rearing of *L. hesperus.* Patana (Patana 1982) developed a system for feeding in Parafilm packets and oviposition in Parafilm packets filled with gel which improved laboratory rearing. Improvements to this diet and to rearing conditions for *L. hesperus* (Cohen 2000a, 2000b) have advanced the mass rearing system for this species.

Mold control in insect diets and colonies is one of the most troublesome components of mass-insect rearing. The conditions of temperature and humidity under which insects are raised are ideal for mold growth. *Aspergillus niger* is one of the most commonly encountered contaminants and one of the most difficult to control (Singh and Bucher 1971, Gifawesen et al. 1975). The *L. hesperus* colony at the Gast (USDA-ARS, BCMRRU, Mississippi State, MS) facility experiences periodic contamination problems with *A. niger* in the diet and in the gel packets used for oviposition. Sikorowski and others established that microorganisms can have detrimental effects on development and health of insects (Kishaba et al. 1968, Singh and Bucher 1971, McLaughlin and Sikorowski 1978, Thompson and Sikorowski 1978, Sikorowski et al. 1980, Sikorowski and Thompson 1984, Sikorowski and Godwin 1985).

It is a common practice to use antimicrobial agents to prevent or suppress microbial contamination of artificial diets for insects. Often antimicrobials are added to artificial diets based more on traditional insect diet recipes rather than taking into account the additive's effect on insect health or the effectiveness of the compound in suppressing microbial growth. Many antimicrobials have been shown to have detrimental effects on some insects (Ouye 1962, Kishaba et al. 1968, Bass and Barnes 1969, Toba et al. 1969, Singh and House 1970). At concentrations above 'safe' levels determined by Singh and House, many antimicrobials delayed development and increased mortality in larval and pupal stages of *Agria affinis* (Singh and House 1970). White fringed beetles, *Graphognathous* spp., have been difficult to rear due to antifungal toxicity problems. Bass and Barnes found that 100% of larvae died within 10 d when a number of different antifungal

[1] E-mail: janalverson@msa-msstate.ars.usda.gov.

agents were used in concentrations high enough for microbial control (Bass and Barnes 1969). These studies and others emphasize the importance of determining the toxicity of an anitmicrobial agent to a particular species of insect before adding it to the diet.

The antifungal agents used in the "NI diet" (Cohen 2000a) currently being used in the production colony of *L. hesperus* at the Gast (USDA-ARS, BCMRRU) facility are formalin and propionic acid. Formalin is a known carcinogen and poses other health risks for employees who are exposed to it during preparation and handling of the diet (Sun 1981, OSHA 2000). Therefore, it would be advantageous to find an alternative antifungal agent that adequately controls mold contamination while not significantly reducing the biological fitness of the insects. Currently, the shelf life of the diet is approximately 2 wk at 4°C and 2 d at 27°C. Shelf life is defined as the length of time the diet may be stored or left on top of a cage (the normal feeding site) before it is noticeably contaminated with microorganisms.

The goal of this research was to identify antifungal agents as alternatives to formalin, for controlling mold (*A. niger*) contamination in the *L. hesperus* diet with minimal negative effects on the health of the insects. We performed bioassays using five antifungal diet additives (benzoic acid, formalin, methyl paraben, propionic acid, and sorbic acid) at three concentrations each, plus a control with no antifungal agents, to determine the impact on the biological fitness of *L. hesperus.* Biological fitness, as used in this research, was determined by measurements of total number of survivors, mean biomass (dry weight) accumulated per cage over the total treatment period, egg production, time to adult emergence, and time to start of egg laying (Cohen 2000a).

Materials and Methods

Insects. *Lygus hesperus* used in these studies were derived from a colony from Biotactics (Riverside, CA), colonized on the "C diet" at the Gast facility for 1.5 yr (A.C.C., unpublished data), and on the new NI diet (Cohen 2000a) for the 21 generations (as of May 2000) previous to this experiment. The NI diet, used in these experiments, was a combination of whole eggs and egg yolks, soy flour, wheat germ, lima bean meal, yeast, and vitamins (Cohen 2000a). Voucher specimens of *L. hesperus* were placed in the Mississippi State University Entomology Museum.

Experimental Protocol. Cages used for all life stages were Rubbermaid 1.7-liter servin'saver rectangular plastic storage boxes (Rubbermaid, Wooster, OH). Cages were topped with organdy cloth (0.4-mm mesh) held tight by the box's snap-on top that had a 15.7 by 26.5-cm opening cut into it. The organdy cloth was changed to a 1.0-mm mesh fiberglass screen when the nymphs were large enough (approximately second instar) to be contained by the larger mesh. Rearing room conditions were 27°C (±1.5°C), 57–65% RH, and a photoperiod of 16:8 (L:D) h. Cages were placed on wire racks, which allowed air circulation and light to reach each cage. The testing colony was treated according to the recommendations of Debolt and Patana (Debolt and Patana 1985) and Cohen (Cohen 2000a) except for the modifications specified here. Egg packets were placed intact, or cut to achieve the correct egg count into cages with shredded paper (0.6 by 28.4 cm) rather than loosely wadded paper towels to reduce cannibalism. Feeding packets and gel packets were made from 20.8 by 10-cm strips of Parafilm, folded and sealed with a heat sealer (Deni Freshlock Turbo II, Keystone, Buffalo, NY) along two sides to create a 10.4 by 10-cm packet with an open top for filling. The first feeding packets provided to newly eclosed nymphs were stretched by hand to facilitate feeding, and one stretched packet was placed inside the box as well as on top of the organdy. Feeding packets on the tops of the boxes were replaced every 48 h and stretched until the nymphs were second instars (appoximately postoviposition day 9). The feeding packets inside the boxes and the egg packets were removed between days 10 and 12, and the organdy was replaced by screening, as nymphal size allowed. Once adults emerged, a 2% Gelcarin (FMC-Food Ingredients Division, Rockland, ME) gel packet was placed on top of the cage for oviposition and changed daily. Previous work (Cohen 2000a) indicated that a higher percentage gel than formerly used (Debolt and Patana 1985) increased egg hatch.

Antifungals and Diet. The diet consisted of NI diet prepared as previously described (Cohen 2000a) except the formalin and propionic acid were omitted. All diet preparations were carried out in a laminar flow hood (formalin was handled in a chemical fume hood). The warm diet (≈45°C) was poured into a sterile glass beaker and the antifungal agents added as follows: no antifungal (control); benzoic acid (4,000, 3,000, and 2,000 ppm); formalin (37% formaldehyde at 1,500, 1,000, and 500 ppm); methyl-paraben (*p*-hydroxybenzoic acid methyl ester at 2,000, 1,500, and 1,000 ppm); propionic acid (1,600, 1,200, and 800 ppm); and sorbic acid (1,600, 1,200, and 800 ppm). The concentrations chosen for testing were based on previous use of these antifungals against *A. niger* (Funke 1983). All chemicals were purchased from Sigma (St. Louis, MO) except the formalin that was purchased from Fisher (Pittsburgh, PA). Sterile water was the solvent for the benzoic acid, propionic acid, and sorbic acid while absolute ethanol was the solvent for the methyl paraben. The pH of the test diets was 5.5 ± 0.2. Approximately 20 ml of diet was dispensed using a syringe into each feeding packet and the tops were heat sealed.

Experimental Design. A randomized complete block design with 16 treatments was used. Treatments were five different antifungal agents tested at three concentrations each, plus a control. Three replications were performed. Blocks consisted of replication in time. Analysis of variance (ANOVA) was performed and mean comparisons were made using least significant difference (LSD) values.

To begin all tests, rearing units consisting of individual cages, described above, were set up and inoc-

Table 1. Effects of antifungal agents on *L. hesperus* biological fitness

Antifungal agent	Concentration, ppm	Total no. of survivors	Total biomass (dry weight in mg)	Eggs per female per day	Days to adult emergence	Days to oviposition
Control		147.0 ± 3.0a	834.1 ± 48.4a	26.77 ± 1.95a	16.3 ± 0.7d	19.0 ± 0.6a
Benzoic acid	2,000	137.0 ± 4.2a	764.2 ± 51.2abc	20.90 ± 2.58abc	17.0 ± 0.0cd	20.0 ± 0.0a
	3,000	146.7 ± 3.0a	773.1 ± 31.1abc	19.73 ± 2.90abc	17.0 ± 0.0cd	20.3 ± 0.9a
	4,000	118.7 ± 19.8a	642.8 ± 70.9abc	18.33 ± 3.43abcd	16.7 ± 0.3cd	20.3 ± 0.7a
Formalin	500	123.3 ± 23.0a	670.7 ± 120.7abc	16.84 ± 3.32bcd	17.3 ± 0.3cd	20.7 ± 0.7a
	1,000	122.7 ± 16.8a	627.2 ± 64.8bc	15.85 ± 5.29cd	17.7 ± 0.7bc	20.7 ± 1.2a
	1,500	136.0 ± 3.5a	680.9 ± 44.4abc	15.75 ± 2.03cd	17.3 ± 0.3cd	20.3 ± 0.9a
Methyl paraben	1,000	117.3 ± 9.2ab	581.6 ± 24.3c	10.92 ± 1.46d	17.7 ± 0.3bc	21.0 ± 0.0a
	1,500	78.0 ± 25.0b	302.8 ± 114.0d	0.92 ± 0.92e	18.7 ± 0.7b	25.0 ± 1.5b
	2,000	23.3 ± 8.7c	91.9 ± 54.3e	0.00 ± 0.00e	21.7 ± 0.3a	26.0b[a]
Propionic acid	800	117.0 ± 14.7ab	684.5 ± 67.1abc	25.13 ± 1.98ab	16.3 ± 0.7d	19.7 ± 0.7a
	1,200	127.0 ± 12.3a	706.0 ± 52.3abc	21.25 ± 3.08abc	17.0 ± 0.0cd	20.0 ± 0.6a
	1,600	126.0 ± 1.5a	696.3 ± 11.7abc	23.67 ± 3.15abc	17.0 ± 0.0cd	19.7 ± 0.3a
Sorbic acid	800	137.7 ± 9.6a	781.1 ± 57.6ab	22.15 ± 4.19abc	17.0 ± 0.6cd	20.0 ± 1.0a
	1,200	128.7 ± 14.3a	772.8 ± 65.8abc	21.82 ± 1.69abc	17.0 ± 0.0cd	20.7 ± 0.3a
	1,600	115.7 ± 18.1ab	656.7 ± 104.1abc	19.70 ± 5.29abc	17.0 ± 0.0cd	20.7 ± 0.9a

Mean ± SE followed by the same letter are not significantly different ($P < 0.05$, PROC ANOVA, LSD test [SAS Institute, 1998]).
[a] Only one egg was laid in this treatment.

ulated with a Parafilm packet containing 200 eggs. All test eggs for each replication were collected within a 2-h oviposition period. The following parameters were measured for each treatment group: (1) total number of survivors; (2) the biomass (dry weight) accumulated per cage over the total treatment period, including adults and deceased nymphs; (3) time to adult emergence; (4) time to start of egg laying; and (5) number of eggs produced by each cage of adults per day. Eggs were collected and counted for 5 d. The experiment was ended 26 d after oviposition (Debolt and Patana 1985). Surviving adults were collected and killed by freezing at −20°C for 4–6 h. Dead nymphs and exuviae were also collected and frozen. Dry weights were obtained after drying both collections at 70°C for 48 h.

Results

Analysis of variance and comparison of means showed some significant differences among treatments. The total number of survivors per treatment cage was significantly lower for the insects raised with the two highest concentrations of methyl paraben in the diet as compared with most other treatments (Table 1). Mean biomass production (in dry weight) per treatment cage was significantly lower for insects raised on diet with medium and high levels of methyl paraben as compared with the other treatments. Methyl paraben and formalin significantly decreased the number of eggs produced per female per day compared with the control. Methyl paraben also had detrimental effects on insect development. The time it took the insects to emerge into adults and the time to oviposition for adults (these insects laid very few if any eggs) was delayed by medium and high concentrations of methyl paraben. Benzoic acid, propionic acid, and sorbic acid did not significantly affect the characteristics of biological fitness measured, as compared with the control, at any of the three concentrations tested for these antifungals.

The eggs produced by each treatment hatched at a rate comparable to the parent *L. hesperus* colony (80–90%, data not shown).

Discussion

The purpose of this research was to measure the effect of various antifungal agents on the biological fitness of *L. hesperus* as defined by the following characteristics: total number of survivors, mean biomass (dry weight) accumulated per cage over the total treatment period, egg production, time to adult emergence, and time to start of egg laying. The research also aimed to identify an alternative to formalin, so that the risk to insectary workers handling the diets could be reduced. Alternative antifungal agents that have less impact than formalin on the characteristics of biological fitness measured in *L. hesperus* include propionic acid, benzoic acid and sorbic acid. Methyl paraben, while widely used with great success with other insect species (Kishaba et al. 1968, Hedin et al. 1974), was shown to have significant negative effects in *L. hesperus.* Ethanol is used as the solvent for methyl paraben and has been shown to interfere with the normal development of some insects (Bass and Barnes 1969, Singh and House 1970). Ethanol, used alone as a dietary additive, is detrimental to *L. hesperus* biological fitness (J.A., unpublished data). Therefore, it was not possible to determine from the new data presented here whether it was the methyl paraben, the ethanol, or the combination, that was detrimental.

In using chemical agents to control diet degradation, there is a fine line dividing toxicity to the microbes that are the targets for suppression and to the insects that are being protected. Selection of an appropriate antimicrobial agent is a crucial and difficult issue. The additives tested in this study were the principal antifungal agents suggested by Funke (Funke 1983) and are currently widely used in insectaries. The mechanisms and ranges of effective pH are important considerations along with concerns about the health

risks to insectary workers. Antimicrobial agents intended to preserve insect diets and colony well being must also meet standards for human safety.

Ideally, in selecting additives to control microbial contamination, it would be useful as a prescreening tool to consider the mode of action of the candidate agents in relationship to their potential to harm insects. For example, the lipophilic acids (including propionate, sorbate, and benzoate) act on fungi to adversely affect active transport capabilities by disrupting cytoplasmic membrane pH (Jay 2000). Without a clear understanding of how such a potential change affects insect cells, however, we must test candidate substances against the insects that the agents are intended to protect. Such tests must be performed on a species by species basis.

Although formalin is a known human health hazard, it still has wide acceptance as an antimicrobial additive in insect rearing. For example, Debolt (Debolt 1982), Debolt and Patana (Debolt and Patana 1985), and Cohen (Cohen 2000a) used formalin in diets for other studies of *L. hesperus.* Although its suggested mode of action, alkylation of nucleic acids and proteins (Russell 1991), would be applicable to both insects and microbes, evidently, at the concentrations used, it is more destructive to the microbes than to the insects.

Many species of insects depend on the activities of symbiotic microorganisms for nutritional or reproductive processes (Bourtzis and O'Neill 1998, Douglas 1998, Moran and Telang 1998). The toxicity of some antimicrobials when used in insect diet may actually be an indirect effect due to the destruction of these beneficial or essential microorganisms. The possible role of symbiotic microorganisms in *L. hesperus* is a question that is being explored in the microbiology laboratory at the Gast facility.

Other investigators have found the rate of larval development to be the most sensitive criterion for measuring the effect of antimicrobials in insect diet (Clark et al. 1961, Ouye 1962, Singh and House 1970). We found delays in development, decreased insect size (mass), decreased fecundity, and an increase in insect death in our tests of different antifungal agents. The bioassays reported here revealed that methyl paraben was the most toxic to *L. hesperus*, followed by formalin, especially in terms of the eggs per female. Both of these antifungal agents should be avoided in rearing *L. hesperus.* The lipophilic acids tested here showed very low levels of toxicity. None caused significant damage to biological fitness, even at high concentrations. Thus, selection of one antifungal agent from among these for future use, will depend on their relative effectiveness against the microbe(s) to be targeted, their relative costs, and the convenience of their use. Human and environmental safety concerns are satisfied by all three of these agents as all of these compounds, in contrast with formalin, are approved for use in human food (Jay 2000). Challenge experiments inoculating artificial diet with *A. niger* are ongoing in the microbiology laboratory at the Gast facility.

Acknowledgments

We thank Lois Connington for excellent technical support in conducting the bioassays, Gay McCain and Brenda Woods for technical support in rearing the *L. hesperus* colony from which research insects were obtained, and Debbie Boykin for statistical advisement. We appreciate the review of an earlier version of this paper by Mike McLaughlin, USDA, ARS, MS, State, MS, and Doug Streett, USDA, ARS, Stoneville, MS. This manuscript is Mississippi Agriculture and Forestry Experiment Station research article No. J-9856.

References Cited

Bass, M. H., and E. E. Barnes. 1969. Toxicities of antimicrobial agents to white-fringed beetle larvae and the effectiveness of certain of these agents against microbial growth. J. Econ. Entomol. 62: 718–719.

Bourtzis, K., and S. O'Neill. 1998. Wolbachia infections and arthropod reproduction. Bioscience 48: 287–294.

Clancy, D. W. 1968. Distribution and parasitization of some *Lygus* species in the Western United States and Central Mexico. J. Econ. Entomol. 61: 443–445.

Clark, E. W., C. A. Richmond, and J. M. McGough. 1961. Artificial media and rearing techniques for the pink bollworm. J. Econ. Entomol. 54: 4–9.

Cohen, A. C. 2000a. New oligidic production diet for *Lygus hesperus* Knight and *L. lineolaris* (Palisot de Beauvois). J. Entomol. Sci. 35: 301–310.

Cohen, A. C. 2000b. A review of feeding studies of *Lygus* spp. with emphasis on artificial diets. Southwest. Entomol. Suppl. No. 23: 111–119.

Debolt, J. W. 1982. Meridic diet for rearing successful generations of *Lygus hesperus.* Ann. Entomol. Soc. Am. 75: 119–122.

Debolt, J. W., and R. Patana. 1985. Lygus hesperus, pp. 329–338. *In* P. Singh and R. F. Moore [eds.], Handbook of Insect Rearing. Elsevier, Amsterdam.

Douglas, A. E. 1998. Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic bacteria *Buchnera.* Annu. Rev. Entomol. 43: 17–37.

Funke, B. R. 1983. Mold control for insect-rearing media. Bull. Entomol. Soc. Am. 29: 41–44.

Gifawesen, C., B. R. Funke, and F. I. Proshold. 1975. Control of antifungal-resistant strain of *Aspergillus niger* mold contaminants in insect rearing media. J. Econ. Entomol. 68: 441–444.

Hedin, P. A., A. C. Thompson, R. C. Gueldner, and R. D. Henson. 1974. Analysis of the antimicrobial agents, potassium sorbate and methyl-*p*-hydroxybenzoate, in boll weevil diets. J. Econ. Entomol. 67: 147–149.

Hedlund, R. C., and H. M. Graham. 1987. Economic importance and biological control of *Lygus* and *Adelphocoris* in North America. USDA Tech. Bull. ARS-64: 95 pp.

Jay, J. M. 2000. Modern Food Microbiology. Aspen, Gaithersburg.

Kishaba, A. N., T. J. Henneberry, R. Pangaldan, and P. H. Tsao. 1968. Effects of mold inhibitors in larval diet on the biology of the cabbage looper. J. Econ. Entomol. 61: 1189–1194.

McLaughlin, R. E., and P. P. Sikorowski. 1978. Observations of boll weevil midgut when fed natural food or on bacterially contaminated artificial diet. J. Invertebr. Pathol. 32: 64–70.

Moran, N. A., and A. Telang. 1998. Bacteriocyte-associated symbionts of insects. Bioscience 48: 295–304.

(OSHA) Occupational Safety and Health Administration. 2000. OSHA regulations (standards-29 CFR), substance

technical guidelines for formalin. U.S. Department of Labor (http://www.osha-slc.gov/OshStd_data/1910_1048_APP_A.html).

Ouye, M. T. 1962. Effects of antimicrobial agents on microorganisms and pink bollworm development. J. Econ. Entomol. 55: 854–857.

Patana, R. 1982. Disposable diet pack for feeding and oviposition of *Lygus hesperus* (Hemiptera: Miridae). J. Econ. Entomol. 75: 668–669.

Russell, A. D. 1991. Priniples of antimicrobial activity, pp. 29–58. *In* S. S. Block (ed.), Disinfection, sterilization, and preservation, 4th ed. Lea and Febiger, Philadelphia.

Sikorowski, P. P., and A. C. Thompson. 1984. Effects of bacterial contamination on development and blood chemistry of *Heliothis virescens.* Comp. Biochem. Physiol. 77A: 283–285.

Sikorowski, P. P., and R. H. Godwin. 1985. Contaminant control and disease recognition in laboratory colonies, pp. 85–105. *In* P. Singh and R. F. Moore [eds.], Handbook of Insect Rearing. Elsevier, Amsterdam.

Sikorowski, P. P., A. D. Kent, O. H. Lindig, G. Wiygul, and J. Roberson. 1980. Laboratory and insectary studies on the use of antibiotics and antimicrobial agents in mass-rearing of boll weevils. J. Econ. Entomol. 73: 106–110.

Singh, P., and H. L. House. 1970. Antimicrobials: 'safe' levels in a synthetic diet of an insect, *Agria affinas.* J. Insect Physiol. 16: 1769–1782.

Singh, P., and G. E. Bucher. 1971. Efficacy of "safe" levels of antimicrobial food additives to control microbial contaminants in a synthetic diet for *Agria affinas* larvae. Entomol. Exp. Appl. 14: 297–309.

Strong, F. E. 1970. Physiology of injury caused by *Lygus hesperus.* J. Econ. Entomol. 63: 808–814.

Sun, M. 1981. Study shows formaldehyde is carcinogenic. Science 213: 1232.

Thompson, A. C., and P. P. Sikorowski. 1978. The effect of bacterial load on amino acids in the boll weevil, *Anthonomus grandis.* J. Invertebr. Pathol. 32: 388–389.

Tingey, W. M., and E. A. Pillemer. 1977. Lygus bugs: crop resistance and physiological nature of feeding injury. Bull. Entomol. Soc. Am. 23: 277–287.

Toba, H. H., A. N. Kishaba, R. Pangaldan, and S. Riggs. 1969. Laboratory rearing of pepper weevils on artificial diets. J. Econ. Entomol. 62: 257–258.

Received for publication 29 May 2001; accepted 29 November 2001.